PRDM1 Is Required for Mantle Cell Lymphoma Response to Bortezomib

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Abstract

Mantle cell lymphoma (MCL) is an aggressive form of B-cell lymphoma with a poor disease-free survival rate. The proteasome inhibitor bortezomib is approved for the treatment of relapsed and refractory MCL and has efficacy in about 30% of patients. However, the precise mechanism of action of bortezomib is not well understood. This report establishes a requirement for the transcription repressor PR domain zinc finger protein 1 (PRDM1, Blimp1) in the response to bortezomib. Bortezomib rapidly induces transcription of PRDM1 as part of the apoptotic response in both cell lines and primary MCL tumor cells. Knockdown of PRDM1 blocks activation of NOXA and inhibits apoptosis, whereas ectopic expression of PRDM1 alone leads to apoptosis in MCL. Two novel direct targets of PRDM1 were identified in MCL cells: MKI67 (Ki67) and proliferating cell nuclear antigen (PCNA). Both MKI67 and PCNA are required for proliferation and survival. Chromatin immunoprecipitation and knockdown studies reveal that specific repression of MKI67 and PCNA is mediated by PRDM1 in response to bortezomib. Furthermore, promoter studies and mutation/deletion analysis show that PRDM1 functions through specific sites in the PCNA proximal promoter and an MKI67 distal upstream repression domain. Together, these findings establish PRDM1 as a key mediator of bortezomib activity in MCL.

Mol Cancer Res; 8(6); 907–18. ©2010 AACR.

Introduction

Mantle cell lymphoma (MCL) is an aggressive form of B-cell non–Hodgkin lymphoma, which makes up 5% to 10% of all human non–Hodgkin lymphomas (1). It involves pre-germinal center B cells present in the mantle zone. MCL is generally characterized by the chromosomal translocation t(11;14)(q13;q32) leading to overexpression of cyclin D1 (2). In addition to cyclin D1 deregulation, MCL is one of the lymphoid malignancies associated with high chromosomal aberrations likely to play an important role in progression of the disease. TP53 mutations (3, 4) and INK4a/ARF deletion are some of the secondary genetic lesions associated with MCL that lead to high proliferation. The majority of MCL patients show a complete or partial clinical response to first-line chemotherapeutic agents mainly based on the CHOP combination or hyperCVAD (2), but relapse is almost certain to result in a median disease-free survival of 3 to 4 years (1).

In 2006, the Food and Drug Administration approved the proteasome inhibitor bortezomib (PS-341, Velcade) for the treatment of relapsed and refractory MCL (3). Bortezomib has also been approved for the treatment of refractory multiple myeloma (4). Bortezomib is a boronic acid dipeptide that binds reversibly to the chymotrypsin-like site in the 20S core of the 26S proteasome (5). Inhibition of the cellular proteasome activity by bortezomib can alter multiple signaling pathways and bring about cytotoxicity. Bortezomib has been shown to inactivate the NFκB pathway in MCL as well as in multiple myeloma (6). However, recent findings have shown that bortezomib is active in MCL with proteasome-insensitive activation of NFκB (7, 8). This indicates that bortezomib must also target other pathways. Bortezomib has been shown to induce apoptosis through the generation of reactive oxygen species and activation of the NOXA pathway in MCL (9). NOXA is a proapoptotic Bcl2 protein that can bind to antiapoptotic Mcl-1 protein, thus releasing Bak from the Mcl-1 complex and promoting apoptosis of the cell. Besides involvement of these pathways, studies in multiple myeloma and some solid tumors such as head and neck cancers have...
revealed that bortezomib can induce apoptosis by inducing endoplasmic reticulum (ER) stress due to the accumulation of misfolded proteins (10, 11). Improperly folded proteins can build up in the ER, leading to activation of the stress signaling pathway known as the unfolded protein response (UPR). UPR is a three-pronged pathway comprising IRE1, PKR-like ER kinase, and activating transcription factor 6 (12). Prolonged ER stress or severe UPR activation leads to cell cycle arrest and induction of apoptosis (13, 14).

PR domain zinc finger protein 1 (also known as PRDM1, Blimp-1, and PRDI-BF1) is a transcriptional repressor required for terminal differentiation of B cells into antibody-secreting plasma cells. During differentiation of mature B cells to plasma cells, PRDM1 represses several key target genes required for maintaining the B-cell phenotype and in maintaining cellular proliferation, such as CHOP, PRAD, SPR-B, β2M, and c-myc (15-19). PRDM1 functions as a repressor by recruiting to the DNA multiple corepressor proteins including the histone H3 methyltransferase G9a (20), the histone deacetylase HDAC2 (21), and the arginine methyltransferase PRMT5 (22). In addition, PRDM1 may displace IRF transcriptional activators through DNA binding site competition at some promoters (23). PRDM1 exists in two isoforms, the full-length PRDM1α and a truncated form, PRDM1β. The truncated PRDM1β, which is abundantly expressed in proliferating myeloma cells and myeloma cell lines, is functionally impaired (24). Recently, PRDM1 expression has been detected in a subset of diffuse large B-cell lymphomas (DLBCL; refs. 25-27). However, inactivating mutations were observed in each case, indicating a tumor suppressor role for PRDM1 (25, 26). Additionally, ectopic expression of PRDM1 in lymphoma cells can induce apoptosis (28). Moreover, induction of PRDM1 transcription in lymphoma cells by anti-IgM treatment induces apoptosis in these cells (29-31). PRDM1 also has been linked to cellular stress responses as a repressor by recruiting to the DNA multiple corepressor proteins (32). Together, these data suggest that PRDM1 is capable of inducing apoptosis in B cells when expressed outside of the plasma cell transition stage.

PRDM1 has not previously been investigated in the context of bortezomib treatment of lymphoma. This report shows that in MCL, PRDM1 is required for the apoptotic effect of bortezomib. Bortezomib induces PRDM1 functions, at least in part, through direct repression of MKI67 and proliferating cell nuclear antigen (PCNA) and inhibits NOXA activity. These findings reveal that PRDM1 is an essential component of the apoptotic response in MCL and a potential important marker for the effectiveness of bortezomib therapy.

Materials and Methods

Cell lines, primary cells, and reagents

The Mino and Jeko-1 MCL cell lines were established and provided by Dr. R. Lai (Department of Laboratory Medicine and Pathology, Cross Cancer Institute and University of Alberta, Edmonton, Alberta, Canada; refs. 33, 34) and maintained in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen). Bortezomib (LC Laboratories) was resuspended in mannitol to make a 10 μmol/L stock solution. Primary MCL samples were maintained in 50% RPMI supplemented with 10% fetal bovine serum and 50% stromal conditioned medium. Stromal conditioned medium was collected from HS5 bone marrow stromal cells cultured in RPMI for 3 days.

Apoptosis assay

Cells were treated with 5 μmol/L bortezomib or, as control, an equal volume of mannitol continuously for 20 hours followed by Annexin V-phycocerythrin staining per manufacturer’s protocol (BD Pharlmeg). Flow cytometry acquisition was done on a FACSCalibur and analyzed with CellQuest software (Becton Dickinson).

Immunoblotting

Whole-cell lysates were prepared using modified radioimmunoprecipitation assay buffer. Lysate from 5 x 10⁵ cells was resolved on 8% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). The primary antibodies used were anti-PRDM1 (Cell Signaling), anti-β-actin (Sigma), and anti-β–(ADP-ribose) polymerase (PARP; Cell Signaling). Secondary antibodies were obtained from Amersham Biosciences.

siRNA knockdown

All siRNAs were purchased from Dharmacon in the Accell modified formulation. Two PRDM1 siRNAs were confirmed to be active and selective and in most experiments were used in an equal mixture of 500 nmol/L each. Delivery of the siRNAs was carried out per manufacturer’s protocol. MCL cell line was incubated with the siRNAs in the Accell siRNA delivery medium for 24 hours, followed by addition of 5 nmol/L bortezomib for 20 hours in the presence of 2% fetal bovine serum. A nontargeting siRNA (Dharmacon) was used as a control in all experiments.

Quantitative mRNA analysis

RNA was isolated from cells using TriZol reagent (Invitrogen) according to the manufacturer’s protocol. One microgram of RNA was DNase treated using RQ1 DNase (Promega), followed by first-strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). In each PCR reaction, 1/20th of the final cDNA reaction volume was used. All primers used are shown in Supplementary Table S1 and were confirmed to produce a single product by melting analysis with an efficiency of 90% to 110% calculated from standard curves. Quantitative PCR reactions were done in a volume of 25 μL, which included 200 nmol/L of each forward and reverse primer and PerC-Taq SYBR Green Supermix for iQ (Quanta). Reactions were run in duplicate using an iCycler and iQ software version 1.0 (Bio-Rad) and duplicates were averaged. Threshold cycle (Ct) for the genes of interest was normalized to the Ct value of a control gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)].
dehydrogenase (GAPDH), β-actin, or GUSB) in each cDNA sample and relative levels of the genes of interest were calculated by the ΔΔCt method (35). Control genes for normalization were selected to have less than 10% variance between experimental conditions.

**Isolation of nascent RNA**

Nascent RNA was isolated as previously described (36). Briefly, nuclei from Mino cells infected with GFP or PRDM1α adenovirus were isolated in an RNase-free buffer composed of 140 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.5% NP40, 1,000 units/mL RNaseOUT (Invitrogen), 1 mmol/L DTT, and 50 mmol/L Tris (pH 8). Extracted nuclei were washed in this buffer three times to remove cytoplasmic RNA, followed by lysis in an RNase-free buffer containing 300 mmol/L NaCl, 1 mol/L urea, 1% NP40, 7.5 mmol/L MgCl2, 0.5 mmol/L EDTA, 1 mmol/L DTT, and 20 mmol/L HEPES (pH 7.6) to isolate histone-bound chromatin. RNA isolation was done as described above. Nascent RNA levels were measured by quantitative reverse transcription-PCR (RT-PCR).

**DNA constructs**

Expression constructs for PRDM1α have been described previously by Ghosh et al. (17). A human MKI67 promoter-luciferase construct consisting of 2,709 bp upstream of the transcription start site and 74 bp downstream was cloned by PCR into the vector PCR2.1 (Invitrogen) using the KpnI and HindIII restriction sites to generate the proximal promoter construct, 2709-MKI67-Luc. A larger MKI67 promoter construct containing the distal PRDM1 site of 2709-MKI67-Luc to create 720-2709-MKI67-Luc. A human PCNA promoter-luciferase construct consisting of 576 bp upstream of the transcription start site and 152 bp downstream was cloned by PCR into the vector PCR2.1 using the XhoI and Smal restriction sites to generate the wild-type PCNA promoter construct, 576-PCNA-Luc. The 576-PCNA-mutPRD-Luc construct was created by site-directed mutagenesis of the 5’-TTTCACTTTACAC-TTT-3’ to 5’-TTTCTCtagACTTT-3’ and was confirmed by sequencing (Mutagenex, Inc.).

**Transfections and luciferase assays**

U2OS cells were transfected using FuGENE transfection reagent (Roche). Transfections for luciferase assays were done with 200 ng of luciferase reporter, 200 ng of PRDM1α plasmid or control pCDNA, and 10 ng of the internal control plasmid pRL-TK. Cells were placed in complete medium for 24 hours before transfection. Incubation with 5 nmol/L bortezomib for 20 hours induced a 3-fold decrease in luciferase activity for the PRDM1α and control plasmids. Transfections were carried out using FuGENE transfection reagent (Roche). Transfections for luciferase assays were done with 200 ng of luciferase reporter, 200 ng of PRDM1α plasmid or control pCDNA, and 10 ng of the internal control plasmid pRL-TK. Cells were placed in complete medium for 24 hours before transfection. Incubation with 5 nmol/L bortezomib for 20 hours induced a 3-fold decrease in luciferase activity for the PRDM1α and control plasmids.
significant elevation of PRDM1α mRNA whereas PRDM1β mRNA was not affected (Fig. 1C). To determine if the PRDM1 present is functionally active, we analyzed the mRNA levels of PRDM1 target genes CIITA and PAX5 (Fig. 1D). Expression of CIITA and PAX5 were repressed by bortezomib treatment. All mRNA levels were assessed by quantitative RT-PCR. The data are presented relative to the control cells after normalization to GAPDH. Columns, mean of three independent experiments; bars, SEM (***, P < 0.002; *, P < 0.05). Relative levels of PRDM1α and PRDM1β mRNA in the control cells were 0.5 and 0.014, respectively.

**FIGURE 1.** Bortezomib treatment induces PRDM1 and apoptosis in MCL cell lines. A, immunoblot analysis for expression of PRDM1 and PARP cleavage in the MCL cell lines Mino and Jeko-1 after treatment with 5 nmol/L bortezomib (Bort) for 20 h. β-Actin is the loading control. Treatment with 5 nmol/L bortezomib for 20 h induces mRNA expression of NOXA (B) as well as PRDM1α (C), but not PRDM1β. D, mRNA levels of PRDM1 target genes CIITA and PAX5 are repressed by bortezomib treatment. All mRNA levels were assessed by quantitative RT-PCR. The data are presented relative to the control cells after normalization to GAPDH. Columns, mean of three independent experiments; bars, SEM (***, P < 0.002; *, P < 0.05). Relative levels of PRDM1α and PRDM1β mRNA in the control cells were 0.5 and 0.014, respectively.

PRDM1 is required for the apoptotic effect of bortezomib

Induction of PRDM1 by bortezomib could be a required event for apoptosis to occur or, alternatively, could be a downstream result of apoptosis. To directly test these two alternatives, PRDM1 expression was blocked during bortezomib exposure and the effect on apoptosis was examined. Mino MCL cells were incubated with two PRDM1-specific siRNAs for 24 hours followed by a 20-hour treatment with 5 nmol/L bortezomib. Apoptosis was analyzed by Annexin V staining and PARP cleavage. As shown in Fig. 3A, the PRDM1-specific siRNA was able to reduce expression of PRDM1α to near basal levels, whereas the nontargeting control siRNA did not block expression. This reduction in PRDM1α was accompanied by a reduction in PARP cleavage (Fig. 3A). Knockdown of PRDM1 also significantly prevented the increase in Annexin V staining.
associated with bortezomib-induced apoptosis (Fig. 3B). To further establish the role of PRDM1 in bortezomib-induced apoptosis, we analyzed proapoptotic genes involved in the bortezomib response. Bortezomib has been shown to upregulate the expression of NOXA in MCL and activate caspase-8 and caspase-9 in multiple myeloma (37). Knockdown of PRDM1 in the presence of bortezomib led to approximately 60% reduction in NOXA expression (Fig. 3C) and approximately 30% reduction in expression of caspase-8 (Fig. 3D) and caspase-9 (Fig. 3E). Thus, the absence of PRDM1 significantly impairs the apoptotic outcome of bortezomib treatment in MCL.

Bortezomib treatment in multiple myeloma induces a stress response because of accumulation of unfolded or misfolded proteins (10). To determine if a similar stress response occurs in MCL cells and if it is dependent on PRDM1, we analyzed the stress response protein XBP1. XBP1 mRNA undergoes unique cytoplasmic splicing in response to ER stress to switch from encoding a negative regulator of UPR to encoding a potent transcriptional activator of UPR (38). Analysis of both splicing isoforms of XBP1 revealed that bortezomib induces XBP1 splicing, but that splicing does not diminish on PRDM1 knockdown (Supplementary Fig. S1). This indicates that bortezomib-mediated ER stress induction alone is not sufficient to induce apoptosis. Together, these data reveal that PRDM1 expression is required for MCL cells to respond to bortezomib.

We next wanted to determine if PRDM1 expression alone is sufficient to promote apoptosis in MCL cells or if additional events induced by bortezomib are required. Because we observed that bortezomib treatment induced only expression of PRDM1α and not that of the truncated PRDM1β, we overexpressed only the full-length PRDM1α form. Mino MCL cells were transduced with a recombinant adenovirus expressing PRDM1α in the absence of bortezomib treatment. Apoptosis was measured by Annexin V staining as well as PARP cleavage after 48 hours of infection. There was an approximately 50% increase in Annexin V staining in cells overexpressing PRDM1α when compared with control cells transduced with an adenovirus expressing only green fluorescent protein (Fig. 4A). To confirm a specific apoptosis effect, we examined PARP cleavage, which is downstream of caspase activation. PARP cleavage was observed only in cells overexpressing PRDM1α (Fig. 4B). This indicates that ectopic expression of PRDM1α in the absence of bortezomib leads to MCL apoptosis. Together, these
findings establish a central role for PRDM1 in the effect of bortezomib and show that PRDM1 is both sufficient and required for the response.

**Identification of direct PRDM1 targets in MCL**

A limited number of direct PRDM1 targets have been identified during B-cell differentiation into plasma cells, including PAX5, CIITA, c-myc, ID3, and Spi-B. In particular, downregulation of Myc by PRDM1 on anti-IgM treatment has been shown to induce apoptosis in Burkitt’s lymphoma cells (30, 31). To determine if bortezomib-induced apoptosis in MCL also involves Myc downregulation, we assessed Myc mRNA changes. Treatment with bortezomib did not affect the mRNA levels of Myc (Supplementary Fig. S2), indicating that other PRDM1 targets must be involved in the bortezomib response.

To identify novel PRDM1 direct targets in B cells, we have used ChIP combined with hybridization to human promoter tiling arrays (ChIP-on-chip). This approach identified multiple targets involved in cell cycle regulation and proliferation, including MKI67 and PCNA. MKI67 codes for the antigen Ki67, which is a proliferative marker and is used as a predictor of survival in MCL. Increased levels of Ki67 in MCL have been associated with deregulation of various cell cycle regulatory components such as overexpression of cyclin D1, HEC, and BUB1B, which are important for the mitotic machinery and downregulation of protein phosphate 2C, which can regulate growth by promoting expression of p53 (39). Furthermore, studies have shown that knockdown of Ki67 leads to cell death in human renal carcinoma cells (40). PCNA codes for PCNA, which is found in the nucleus. PCNA is a multifunctional protein that plays a role in both DNA replication and DNA repair (41). It is a subunit of DNA polymerase δ and can interact with p21 to pause replication while allowing DNA repair to occur. In addition, loss of PCNA can lead to a p21-mediated growth arrest in lung epithelial cells exposed to hyperoxia (42). Moreover, cells that lack PCNA expression undergo apoptosis. Studies have shown that in the WST-knockout mouse model, PCNA is absent from the thymus and spleen leading to reduced size of the tissues and expression of apoptotic markers in these tissues (43). Together, these observations suggest that PCNA and Ki67 may be functionally important targets of PRDM1.

Sequence analysis of the MKI67 and PCNA promoter regions suggested potential binding sites for PRDM1 at a distal enhancer region of MKI67 (−4,290 to −3,594 bp) and the proximal promoter of PCNA (−818 to −235 bp, relative to the transcription start site). Binding of PRDM1 at these sites was determined by ChIP and quantitative PCR (Fig. 5A). Mino MCL cells treated with 5 nmol/L bortezomib showed a significant binding of PRDM1 at both MKI67 and PCNA. The known PRDM1 targets PAX5 and CIITA also showed similar levels of PRDM1 binding as expected. This binding was specific because no signal was detected at the HLA-DRA promoter. PRDM1 is known to repress its targets, in part, by recruiting the histone deacetylase HDAC2 and the histone methyltransferase G9a (20, 21). This results in a loss of acetylation of the histones and specific dimethylation of H3K9, both of which are associated with gene silencing. As shown in Fig. 5B, bortezomib treatment led to a decrease in histone H3 acetylation on the MKI67 and PCNA promoters. A similar decrease in acetylation was observed for PAX5 and CIITA whereas the control promoter HLA-DRA was not changed. The change in acetylation was accompanied by increases in dimethylation of H3K9 residues consistent with PRDM1-mediated silencing (Fig. 5C).

**PRDM1 regulates the promoter activity of PCNA and MKI67**

Because PRDM1 regulates its targets at the level of transcription, we cloned the human MKI67 and PCNA promoters to assess if PRDM1 can repress their promoter activity. A PCNA promoter spanning 576 bp upstream of the transcription start site and 152 bp downstream was cloned into a luciferase reporter plasmid. A potential PRDM1 binding site was identified by sequence homology at position −296 and was selectively mutated to create a PCNA mutant promoter construct. Luciferase activity of the wild-type PCNA promoter was reduced by 60% in the presence of PRDM1α (Fig. 6A). In contrast, PRDM1α failed to repress the mutant PCNA promoter construct. This indicates that PRDM1 functions specifically through this DNA element to suppress PCNA.

A similar study was carried out on the human MKI67 promoter. The PRDM1 binding site is located about
3.5 kb upstream of the transcription start site. A basal promoter construct was created spanning 2,709 bp upstream of the transcription start site and 74 bp downstream. In addition, a 720-bp fragment containing the distal PRDM1 binding site was cloned upstream of the proximal promoter. Analysis reveals that the proximal promoter construct was active but not altered by the presence of PRDM1 (Fig. 6B). In contrast, when the distal region containing the PRDM1 binding site was present, the MKI67 promoter activity was repressed by approximately 40%. This indicates that PRDM1 functions through specific DNA elements present in both the PCNA and MKI67 promoters.

PRDM1 directly represses endogenous target genes at the level of transcription

The ChIP assay revealed that PRDM1 can directly bind to the novel target genes MKI67 and PCNA in MCL cells. Moreover, luciferase promoter activity for these promoters is repressed by approximately 40%. This indicates that PRDM1 functions through specific DNA elements present in both the PCNA and MKI67 promoters.

RNA levels for MKI67 and PCNA were analyzed. Treatment of Mino and Jeko-1 MCL cells with 5 nmol/L bortezomib for 20 hours induces PRDM1 (Fig. 1A) along with approximately 50% to 80% repression of both MKI67 and PCNA at the mRNA level (Fig. 7A). Knockdown of PRDM1 expression leads to a derepression of MKI67 and PCNA mRNA (Fig. 7B). This provides strong evidence that PRDM1 expression is required for the repression of MKI67 and PCNA in response to bortezomib. To further confirm that PRDM1 represses MKI67 and PCNA at the transcriptional level, we analyzed the nascent RNA levels for these genes after PRDM1α overexpression. Nascent RNAs are those RNAs that are still in the process of being transcribed and are an accurate measure of endogenous transcriptional activity (36). The nascent RNA was purified from nuclei after extensive washing to remove the released transcripts and were quantified by quantitative RT-PCR. The nascent RNA levels for MKI67 in MCL cell line expressing PRDM1α showed a 70% to 80% repression (Fig. 7C) when compared with control cells transduced with a control

FIGURE 5. Chromatin immunoprecipitation of PRDM1 and associated epigenetic marks at the MKI67 and PCNA promoter regions. A, ChIP using the PRDM1 antibody in Mino MCL cells treated with 5 nmol/L bortezomib for 20 h. PRDM1 binding at the MKI67 and PCNA promoters was significantly higher in bortezomib-treated cells compared with control cells (untreated). PAX5 and CIITA are positive controls for PRDM1 binding, and HLA-DRA (DRA) is a negative control. Binding was quantified by quantitative PCR and is presented as relative occupancy (antibody-specific signal over signal obtained with nonspecific IgG antibodies). Columns, mean of three independent experiments; bars, SEM (**, P < 0.002; **, P < 0.03; *, P < 0.05). B, ChIP analysis of acetylation on H3K9. The conditions are as described for A, except an antibody specific to acetylated H3 was used. Acetylation is significantly decreased concordant with PRDM1 binding. C, ChIP analysis of H3K9 dimethylation levels. The conditions are as described for A, except an antibody specific to dimethylated H3K9 was used. Dimethylation is significantly increased concordant with PRDM1 binding.
GFP-expressing adenovirus. A similar extent of repression was observed for PCNA at the nascent RNA level (Fig. 7C). Overexpression of PRDM1 did not repress the nascent RNA levels of PU.1, which is not a PRDM1 target. These data confirm that PRDM1 can specifically repress endogenous MKI67 and PCNA at the level of transcription in MCL.

To further assess that repression of MKI67 and PCNA by PRDM1 is functionally relevant, we analyzed the protein levels of these genes. Immunofluorescence staining reveals that bortezomib treatment dramatically reduces both PCNA and Ki67 levels. However, siRNA knockdown of PRDM1 expression prevents the loss of endogenous PCNA and Ki67 proteins in response to bortezomib (Fig. 8).
FIGURE 8. PRDM1 is required for the bortezomib-mediated suppression of PCNA and Ki67.

A, immunofluorescence staining of Mino and Jeko-1 MCL cells treated with bortezomib in the presence or absence of siRNA-mediated knockdown of PRDM1. PCNA protein detected by FITC (green), Ki67 protein detected by Alexa594 (red), and 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (blue) are shown from a representative panel. Magnification, ×63.

B, quantitative analysis of the immunofluorescence for Mino. Data were collected from two independent experiments with duplicate slides and at least five individual images per slide were analyzed for each condition by automated Difiniens software (*, P < 0.05).
Discussion

MCL continues to have a poor prognosis and a low disease-free survival rate. The proteasome inhibitor bortezomib was approved for the treatment of relapsed and refractory MCL in 2006 (3) and shows promise with an overall response rate of 32% (44). However, the specific mechanisms by which bortezomib is cytotoxic to MCL remain unclear, which is a significant barrier to understanding how to improve or tailor bortezomib therapy (6, 8–11). The findings presented in this report now show that bortezomib induction of apoptosis in MCL is accompanied by and requires induction of the transcriptional repressor protein PRDM1. Induction of PRDM1 occurs at the level of transcriptional activation. Protease-mediated regulation of transcription has been reported in several systems and shown to affect activation, elongation, as well as chromatin structure (45, 46). In addition, PRDM1 has a PEST domain homology region that could target it for proteasomal degradation. Although our studies have not excluded an additive effect of protein stabilization, clearly, activation of PRDM1 transcription is required for MCL cells to respond to bortezomib.

NOXA is a key proapoptotic sensor protein that leads to an increased activity of the mitochondrial apoptotic pathway by activating BAK. Several recent studies have clearly linked NOXA to the bortezomib response in both sensitive and intrinsically resistant MCL cells (8, 9). Bortezomib selectively induced expression of NOXA but not that of other BH3-only proteins. Importantly, siRNA knockdown of NOXA resulted in an approximately 70% reduction of apoptosis, indicating that NOXA is a key step in the bortezomib response. Our findings indicate that induction of NOXA is dependent on induction of PRDM1. siRNA knockdown of PRDM1 not only inhibited apoptosis by approximately 70% but also significantly blocked NOXA expression. This is consistent with a central role for NOXA but now also places PRDM1 activation upstream of NOXA induction in the response to bortezomib. The mechanism of NOXA induction is not understood; however, it is unlikely that PRDM1, a repressor protein, directly activates the NOXA promoter. There is no evidence for PRDM1 binding to the NOXA promoter, rather PRDM1 is more likely to suppress additional gene(s) whose absence permits NOXA activation, although this remains to be resolved.

Recent studies in DLBCL have identified inactivating mutations in the PRDM1 gene, indicating a tumor suppressor role for PRDM1 (25, 26). The PRDM1 protein has two isoforms, PRDM1α and the truncated PRDM1β, which are transcribed from alternative promoters. PRDM1β has been shown to be highly expressed in myeloma cells and is associated with impairment of PRDM1 repressive activity (24, 47). Additionally, expression of PRDM1β in DLBCL has been associated in one study with chemoresistance and poor disease outcome (48), indicating an impaired tumor suppressor activity of the β isoform. Related observations have been made in human myeloid leukemia cell lines in which cellular stress led to expression of PRDM1α but not PRDM1β (32). Similarly, our findings show that bortezomib exposure leads to selective expression of the PRDM1α isoform in MCL and support the idea that the PRDM1α isoform is the functionally active form and that the PRDM1β isoform arises to potentially squelch the activity.

To date, mutations of PRDM1 have not been reported in MCL. MCL arises from pre-germinal center B cells, a stage preceding normal PRDM1 expression, which first occurs in late germinal center B cells. Thus, the abundant PRDM1 expression in MCL induced by bortezomib is out of its normal physiologic context, which may facilitate the apoptotic outcome. This is consistent with our observation that ectopic expression of PRDM1α alone in MCL promotes apoptosis. Interestingly, a recent report has suggested that long-term exposure of MCL cell lines to low doses of bortezomib to induce drug resistance is accompanied by a plasmacytic-like gene expression pattern, including PRDM1 expression (49). This supports our findings that bortezomib induces PRDM1, but suggests that suboptimal exposure to bortezomib can induce a partial differentiation program. It will be interesting to determine if these resistant lines acquire expression of the PRDM1β isoform, similar to myeloma cells, or if they acquire PRDM1 mutations similar to DLBCL to abrogate normal PRDM1 activity.

A very limited number of genes have been identified to be directly regulated by PRDM1. The majority of these genes are transcription factors related to B-cell development and differentiation. Our discovery that PRDM1 directly represses two genes required for proliferation establishes a novel role for PRDM1 in regulating cell growth and viability. Furthermore, downregulation or knockdown of either PCNA or MKI67 in tumor cells can induce apoptosis (40, 42). Thus, PCNA and MKI67 may be highly potent targets of bortezomib-induced PRDM1 by inhibiting proliferation as well as inducing apoptosis in MCL. Little information is available about the transcriptional regulation of MKI67, and this is the first report that PRDM1 directly suppresses MKI67. However, a recent finding in sebaceous glands has shown that cells expressing PRDM1 are devoid of Ki67 expression (50). Similarly, microarray studies in B cells have shown an inverse correlation between PRDM1 expression and PCNA (16). Moreover, the significance of our data may not be limited to the response of MCL to chemotherapeutic agents but may also have significance in T-cell homeostasis. PRDM1 has been shown to play a role in maintaining T-cell homeostasis by increasing apoptosis of effector and memory T cells (51). In these studies, there were no substantial changes observed in the key survival regulatory proteins such as myc, Bcl-2, Bcl-xL, and CTLA4. It will be important to determine if PRDM1 can directly suppress MKI67 and PCNA in these T cells and induce apoptosis. It may also be possible that MCL tumor cells may have a unique response to PRDM1 when exposed to bortezomib compared with normal cells. Further defining the global network of PRDM1-regulated genes in multiple cell types will be important to shed light on this question.
In conclusion, this is the first study identifying an important role for PRDM1 in bortezomib-induced apoptosis of MCL. We propose a mechanism of action in which PRDM1 induced by bortezomib leads to direct repression of the proliferation markers MKI67 and PCNA, inducing apoptosis in these cells. Finally, our data support that approaches to directly target induction of PRDM1 may be an attractive means to enhance current therapies for MCL patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

We thank the H. Lee Moffitt Cancer Center Flow Cytometry, Analytical Microscopy Core, and Microarray Core Facilities.

Grant Support

NIH, National Cancer Institute grant CA680990 (K.L. Wright). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 04/01/2010; accepted 05/07/2010; published OnlineFirst 06/08/2010.

www.aacrjournals.org Mol Cancer Res; 8(6) June 2010 917

Published OnlineFirst June 8, 2010; DOI: 10.1158/1541-7786.MCR-10-0131

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